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13. ABSTRACT *(Maximum 200 Words)*

Osteopontin, a secreted phosphoprotein, is a major modulator of the motility of several cell types including macrophages, osteoclasts and tumor cells. Through its interaction with integrins and CD44v, osteopontin can induce metalloprotease, inhibit apoptosis, inhibit NO production and induce cytokine secretion. We have recently isolated a hexa peptide from osteopontin that is chemotactic to tumor cells. Antibodies raised against this peptide neutralize the chemotactic response of tumor cells to osteopontin *in vitro* and *in vivo*. Our hypothesis is that osteopontin or chemotactic peptides released during bone remodeling attract circulating breast tumor cells expressing specific CD44v splice variants. We have isolated a peptide analogue of the chemotactic domain (PepL) that inhibits tumor cell migration, induces Nitric Oxide production and activates apoptosis in tumor cells. We now report that the chemotactic domain of OPN induces the activation of FAK, Protein Kinase Cβ (PKCβ) and PI-3 kinase, while PepL inhibits the activation of FAK, inhibits the activation of PKCβ, but can activate PKCζ. Further mutants of OPN lacking the chemotactic domain cannot stimulate the chemotaxis of tumor cells nor can they activate protein kinase Cβ. However, these mutants can activate PI3 kinase and FAK. We concluded that OPN mediates tumor migration by regulating, FAK, PI3 Kinase and PKCβ. We further conclude that PepL mediates its anti-tumor activity by inhibiting the activation of FAK and the activation of PKCζ. We Further conclude that activation of PKCζ might result in the activation of NKκb and the induction of iNOS.

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INTRODUCTION

Several cell surface molecules that modulate cell-cell and cell-matrix interactions have been linked to metastatic spread. These cell surface receptors, which include the laminin receptor, cadherins, Ig superfamily of receptors, integrins, selectins ICAMS and CD44 (Steller and Stevenson *et al.*, 1993),. may participate at specific steps in the metastatic spread of tumor cells and influence cell migration, invasion, spreading and cell growth. Blocking one of these steps may result in inhibition of metastasis. Breast tumors metastasize to bone and brain more frequently than any other organ. The factors responsible for the frequent metastasis of breast tumors to bone are currently unknown. The CD44 gene encodes a transmembrane protein which is expressed as a family of molecular isoforms generated from alternative RNA splicing. Standard form of CD44 is expressed in many normal tissues, whereas the expression of variant isoforms of CD44 (CD44v) is restricted (Tolg *et al.*, 1993). Some CD44v isoforms, which may regulate the homing of lymphocytes and macrophages after antigenic stimulation *in vivo,* have also been implicated in the metastatic spread of tumor cells. Transfection of CD44 isoforms into non-metastatic tumor cells transforms these cells into metastatic ones. Furthermore, antibodies raised against CD44 inhibit the metastatic spread of a variety of tumors (Arch, 1992). CD44v is expressed on the surface of metastatic breast tumors and it is currently being investigated as a possible prognostic tool for patients diagnosed with breast cancer. Recent reports have correlated the expression of CD44v (containing exon v3,v5,and v6) on breast tumors (Kaufman *et al.*, 1995) with metastasis and poor prognosis in patients with breast cancer. It is still unclear which molecule(s) CD44v is interacting with and at what stage in the metastatic cascade is CD44 critical. Osteopontin, a secreted phospho-glycoprotein secreted by bone cells, promotes the chemotaxis of macrophages and tumor cells through CD44v receptor and that the migration of these cells to OPN can be inhibited by antibodies raised against either OPN or CD44. Our hypothesis is that osteopontin or chemotactic peptides released from bone during remodeling attract breast tumor cells expressing specific CD44 splice variants to migrate out of the capillary bed of bone marrow. The extravasated tumor cells can then attach to matrix bound osteopontin resulting in the induction of MMP2 and MMP9 which may further facilitate the invasion and growth of tumor cells within the bone micro-environment by removing connective tissue barriers and releasing matrix bound cytokines.

Invasion though a three-dimensional extracellular matrix is a coupled event requiring cell attachment, detachment and localized degradation of the matrix in the direction of movement. This coordinated process requires the synergistic interaction among several types of receptors with different molecules of the extracellular matrix. This grant has **three Specific Aims**. In the **first Aim** we will examine the invasion of the CD44 transfectants into marrow stroma and MC3T3E1 cell cultures. A chemotactic domain on OPN was defined. In the **second aim** we will examine the role of this chemotactic domain on the migration of breast tumor cells and determine which amino acids within the chemotactic domain are essential for chemotaxis. Once an essential amino acid has been identified, we will mutagenize this amino acid in OPN and determine the biological consequences of the mutagenesis. In the **third Aim** we will assess the *in vivo* role of CD44 splice variants in bone specific metastasis and test whether the anti-osteopontin antibodies will neutralize the metastasis of MDA-MB-231 and MDA-MB-453/CD44 transfectants to bone.

(2) **BODY:**

The approved statement of work is appended.

Technical Objective 1: **CD44 expression and invasion into Bone marrow and MC3T3E1 Cultures.** Tasks 1a-e have been completed and included in first year report.

Technical Objective 2**: Effect of Chemotactic Peptide on the migration of MDA-MB-231 and transfected clones.** Tasks 2a-2c have been completed. 2d-2g are in progress

Construction of OPN mutants: To evaluate the contribution of the chemotactic domain to the biology of osteopontin, we adopted a two-tier strategy. In the first tier, we constructed several forms of OPN, lacking the chemotactic domain. These constructs are presented in figure 1. OPN 286 was constructed by cloning amino acids (1-285) into PCDNA3.1 (Invitrogen Corporation, Carlsbad, California).. OPNdcc was constructed by cloning amino acid 291-314 in frame into the 5' end of OPN286 cDNA. The resulting protein has the LVVD replaced by LRP. The resulting OPN286 and OPNdcc clones were separately transfected into LL64, a small cell carcinoma cell line established from the OPN knockout mouse. Stable transfectants over-expressing either OPN286 or OPNdcc were used to purify OPN286 or OPNdcc protein by a modification of our OPN purification method (Ashkar et al., 2000). In the second tier we mutagenized the aspartate in the sequence LVVDPK into an alanine or a glycine. These clones are currently being sequenced and characterized.

OPN

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYD**GRGDS**VVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDM**LVVDPKS**KEEDKHLKFRISHELDSASSEVN

OPN286

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDMRA

OPNDCC

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDMARPKSKEEDKHLKFRISHELDSASSEVN

Figure 1: Sequence of Human OPN, OPN 285 and OPNdcc: RGD integrin binding sequence and LVVDPK chemotactic sequence are in Bold.

Regulation of Tumor cell migration by OPN285 and OPNdcc. OPN285 and OPNdcc were tested for their ability to induce tumor cell chemotaxis and cell spreading. The results summarized in table 1 clearly show that both OPN286 and OPNdcc can support the spreading of MDA-MB-231 cells. However, neither protein can induce the chemotaxis of these cells.

Protein	Chemotactic Index	% Spread
Control	1 ± 0.2	10% ±3
OPN	**10.5 ± 2.2**	**87% ± 18**
OPN286	0.9 ± 0.2	**96% ± 21**
OPNdcc	1.1 ± 0.3	**91% ± 11**

TABLE 1: Chemotaxis of tumor cells to OPN286 and OPNdcc.

A) Directed migration of cells was determined in multi-well chemotaxis chambers. Two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5 μg fibronectin. 2 X 10^5 MDA-MB231 cells were added to the upper chamber and incubated at 37° C in the presence or absence of osteopontin in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically. Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. Data are expressed as migratory index (cells migrating in response to osteopontin/cells migrating in response to buffer). Values are expressed as mean ± standard error, numbers in bold are significantly different from control values with $p < 0.05$ or better. All assays were done in triplicates and are reported as mean ± standard error.

B) 24-well plates were coated overnight at 4°C with 10 μg/ml of the indicated ligand then blocked for 1 h at room temperature with 10 mg/ml BSA in PBS. To preserve the integrity of adhesion receptors MH-S monocytic cells were harvested from subconfluent cultures by non-enzymatic cell dissociation solution (Sigma, St Louis MO). Cells were washed twice with PBS and resuspended at a concentration of 1 X 10^5 cell/ml of sterile Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1% BSA and 1 mM sodium pyruvate. 5 x 10^4 cells were incubated in each well and, after 1 h at 37°C, the wells were washed 3 times with 0.5 ml PBS to remove non-adherent cells, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 hour then stained with toluidine blue and hematoxylin. The total number of attached or spread cells in each well were counted microscopically using a Nikon Eclipse microscope equipped with a Sony digital Camera. Total number of attached or spread cells were quantitated using Optima 5.2 image analysis system. Each experiment was done in triplicates and is reported as mean ± standard error. To minimize variability inherent to cell attachment studies we scored cells as attached only when a defined nucleus was observed accompanied by a transition from round to cuboidal cell morphology. Round cells that are loosely attached with no defined nucleus were scored as non-attached. These cells can be removed with repeated washes. The viability of the cells was measured before and after the termination of the experiments and only data from experiments with greater than 95% cell viability were used. Further, under the conditions used in these experiments, cell attachment was temperature dependent, inhabitable by trypsin treatment and was not affected by inhibitors of protein synthesis or secretion. Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area.

In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Modulation of PI3 kinase and FAK PKC activity by the chemotactic peptide (PepA) and its antagonist PepM

A proximal mediator of osteopontin dependent signal transduction through integrin receptors is the 125 kD focal adhesion kinase (Hruska et al. 1995) which associates with integrin $\alpha_{\varsigma}\beta_3$ and, in synergy with the cytoplasmic tail of integrin α_{ς}, activates pp60c-Src (Chellaiah et al. 1996). Two downstream processes ensue which both modulate the cytoskeleton. Tensin and paxillin may be activated by p125 FAK and pp60c-Src (Lopez et al.,1993). Src also activates phosphoinositide 3-hydroxyl kinase by tyrosine phosphorylation of the Src homology 2 domain in the p85 subunit (Hruska et al. 1993). PI 3-kinase may regulate the arrangement of actin filaments through gelsolin in a process that is inhibitable by wortmannin (Chellaiah/Hruska 1996). While both pathways have been associated with cell motility and spreading, their individual contributions to these processes are not known.
Many protein kinases require phosphorylation within their activation loops in order to express full catalytic potential. Such activation loop phosphorylations are also important for protein kinases regulated acutely by allosteric effectors. This is exemplified by PKC, where the Ca2+/diacylglycerol (DAG)-dependent isotypes PKC α and PKC β display an absolute requirement for phosphorylation in their respective activation loops. Suppression of phosphorylation at these PKC sites *in vivo* correlates with the induction of apoptosis in certain cell types (Garcia-Paramio et al., 1998), demonstrating the essential role of this phosphorylation *in vivo*. One potential kinase that has been implicated in the phosphorylation of PKC is the PtdIns(3,4,5)P3 dependent kinase PDK1. PDK-1 has been shown to phosphorylate PKC within their activation loop (Good et al., 1998). The effect of PDK1 is PI 3-kinase-dependent, and is inhibited *in vivo* by LY294002. PKC, therefore, is controlled through a PI 3-kinase pathway, operating through PDK1-dependent phosphorylation of activation loop sites in the PKC isotypes.

Earlier studies have identified a role for PKC in cell crawling. Treatment of the colon carcinoma cell line HT29-D4 with PMA increased the rate of cell spreading and induced the migration of these cells towards purified matrix proteins in Boyden chamber-based haptotaxis assays. HT29-D4 cell haptotaxis was a direct consequence of PKC activation and not secondary to quantitative or qualitative changes in the cell surface integrins (Rigot et al. 1998). In crawling T cells, triggered via cross-linking of integrin LFA-1, two PKC isoenzymes, β(I) and δ, are targeted to the cytoskeleton with specific localization corresponding to the microtubule-organizing center and microtubules. Cells of a PKC-β–deficient clone derived from the parental PKCβ-expressing T cell line can neither crawl nor develop a polarized microtubule array upon integrin cross-linking. However, their adhesion and formation of actin-based pseudopodia remain unaffected (Volkov et al. 1998).

Since OPN regulates both FAK and PI3 kinase, we examined the effect of the chemotactic domain and its antagonist on the activation of FAK, PI3 kinase and PKC. The results indicate that OPN, OPN286 and OPNdcc can activate FAK and PI3 Kinase, while PepA (Chemotactic peptide) and PepL can not. Further, OPN and PepA can activate PKCβ but not the zeta or gamma isoforms, while PepL only activates the zeta isoform. OPN286 and OPNdcc failed to activate any of the PKC isoforms tested. (Figure 2).

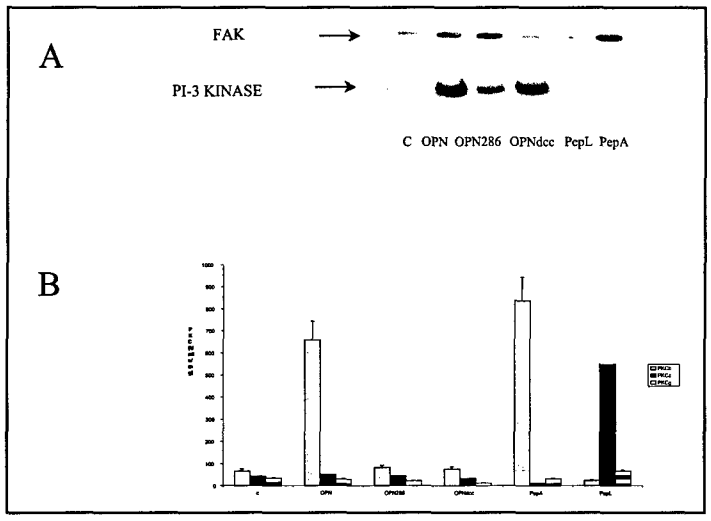

Figure 2 Regulation of Signal transduction by OPN and its analogues.
A) 1 million MDA-MB-231 cells were incubated with either OPN (5 mM), OPN286 (5 mM), OPNdcc (5 mM), PepA (5 mM) or Pep L(5 mM). After 12 hours the cells were harvested and lysed in 1 ml buffer A (10 mM Tris-HCl buffer, pH 7.2, containing 300 mM sucrose, 100 mM KCl, 1% Triton X 100 5 mM $MgCl_2$, 10 mM EGTA, 0.1 mM sodium orthovanadate, 0.1 M ε-amino-n-caproic acid, 5 mM benzamidine, 1 mM p-hydroxymercuribenzoate, 5 mg/l pepstatin, 1 mg/l leupeptin) at 4°C. After 10 min, fractions containing 2 mg protein were pre-cleared by incubation with 100 µl of insoluble protein A at 4°C for 1 h. After centrifugation at 5000 x g for 10 min., the resultant supernatant was incubated with 0.1 mg of rabbit polyclonal antibody raised against either the p85a subunit of PI-3-Kinase (Upstate Biotechnology, Lake Placid, N.Y.) or FAK monoclonal antibody (BD Transaction Laboratory, Franklin Lakes, NJ) . The immune complexes were collected by incubation with 10 µl insoluble protein A for 1 h at 4°C followed by centrifugation at 5000 x g for 10 min. The protein A-immunocomplexes were washed 5x with lysis buffer, then once with 20 mM Tris-HCl. The immune complexes were released from the protein A beads by boiling the beads in 20 ml of SDS sample buffer containing 0.1% fresh 2-mercaptoethanol. The samples were resolved by on an 8% SDS-polyacrylamide gel and then transferred onto ECL-membrane by semi-dry blotting as described by the manufacturer. Phosphorylation of PI-3-kinase and FAK were assessed by probing the membranes with anti-phosphotyrosine (BD transduction Laboratory, Franklin Lakes, NJ).

B) One million tumor cells were treated as above. The cells were harvested and lysed and the activity of protein kinase C was measured with a kit obtained from Panvera. This assay distinguishes isoforms of protein kinase. We performed two separate experiments with duplicate samples.

Since PepL cannot induce PKCβ, We examined the effect of PepL on OPN induced PKCβ. The results show that in 1umols of PepL completely abolishes the induction of PKCβ by OPN (581± 23 units/mg to 21 ± 11 units/mg in the presence of 1 umol of pepL). These results suggest that PepL is a potent inhibitor of pkcb activation and explains in part it anti migratory properties. Based on the results collected so far, we propose that pepL could be a potent anti-tumor agent and we wish to test it *in vivo*.

Technical Objective 3: **Testing the anti-metastatic potential of anti chemotactic domain antibody *in vivo* and testing of novel CD44 cell lines for their metastatic potential *in vivo*.**

Task 3a-3f are in progress. We are currently analyzing the histology from these experiments

We have constructed the following cells line to

- MB-231b: human adenocarcinoma cell line cloned from a bone metastasis in nude mice of the parental cell line MB-MDA-23; CD44s, Cd44E, CD44 (v3-v6), integrin alpha v, 4, 6, b1, b5, b3, Sialyl lewis, ICAM, Muc-1, HER2, p53, ER^-
- MDA-MB-453. human adenocarcinoma cell line $CD44^-$ Sialyl lewis, ICAM, Muc-1, HER2, p53 integrin a2, av, a6(low), b1, b5, b3(low), cell lines that do not metastasize *in vivo* :
- MB-453 (CD44s): Tumor cells expressing the standard form of CD44. Detected in all breast tumors but not normal breast tissue (Fichtner et al., 1997).
- MB-453 (CD44E): Tumor cell line expressing the epithelial form of CD44. Detected in 85% of breast tumors (Fichtner et al., 1997)
- MB-453 (CD44v (3,5,6): Tumor cell line expressing CD44 splice variant containing exons 3,5 and 6 that has been linked to metastasis of breast tumors and poor prognosis (Fichtner et al., 1997).
- MB-453i: A clone of MB-453 (CD44v (3,5,6) that expresses integrin b3. This cell line is highly metastatic.
- MB-453 (CD44v (6,7) Tumor cell line expressing CD44 splice variant containing exons 5,6. A splice variant more prevalent in estrogen receptor negative tumors (Fichtner et al., 1997).
- MB-453 (CD44v (9, 10) Tumor cell line expressing CD44 splice variant containing exon 9, 10. Expressed in some tumors and correlates with histological grading (Fichtner et al., 1997).

We are currently assessing the signals induced by OPN and its mutants, as well as, pepA and PepL in these cell lines

(3) **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of **key** research accomplishments emanating from this research.

- Mutants of OPN lacking chemotactic domain cannot induce migration of CD44(v3-v6) tumor cells.
- Chemotactic domain of OPN induces the activation of PKCβ.
- PepL, which antagonizes tumor migration, induces Nitric oxide, and inhibits PKCβ.activation.
- Treatment of tumors with PepL results in the Induction of IL-12 by resident macrophages.
- PepL may by a good candidate for anti-tumor therapy.

(4) **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes to include:

1- Weber, G.F., and **Ashkar, S.** (2000). Stress Response Genes - The Genes That Make Cancer Metastasize. Journal of Molecular Medicine. 78: 404-408.

2- Weber, G.F and **Ashkar, S.,**. Molecular Mechanisms of Tumor Dissemination in Primary and Metastatic Brain Cancers (2000). Brain Research Bulletin. 53:421-424.

(5) CONCLUSIONS:

In the second year of the grant, we gained unique insight into the molecular biology of tumor migration and metastasis. We are beginning to decipher the signal transduction pathways that are operating in mediating tumor metastasis. Based on our results and those of others, we propose that tumor metastasis is mediated by a complex of molecules that form a functional unit that transmits information from the environment and regulates tumor behavior. This invasion complex appears to mimic, in part, the migration complex on immune cells and function in a manner similar to it. Further the tumor invasion complex or invasome makes a novel target for treatment of advanced cancer.

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(7) **APPENDICES:**

Statement of Work

Technical Objective 1: **CD44 expression and invasion into Bone marrow and MC3T3E1 Cultures**

Task 1a: (Month 1-4) Analyze the invasiveness of CD44 cell lines (described above) into MC3T3E1 and Marrow stromal cultures. Correlate expression of specific CD44 with attachment and invasion

Task 1b: (Month 4-6) Analyze the effect of CD44 antibodies (both general and exon specific) on the invasiveness of the CD44 cell lines.

Task 1c: (Month 7-9) Analyze the effect of osteopontin antibodies (polyclonal and monoclonal) on the invasiveness of the CD44 transfected cell lines.

Task 1d: (Month 10) From the above data Examine the physical and temporal relationship between the expression of CD44 splice variants and invasiveness into marrow and bone cultures.

Task 1e: (Month 10-14) Examine the role of CD44 splice variants in the attachment and migration into endothelial cell culture.

Technical Objective 2: **Effect of Chemotactic Peptide on the migration of MDA-MB-231 and transfected clones.**

Task 2a: (Month 1) Synthesize chemotactic peptide and peptides altered in one or more amino acid.

Task 2b (Month 2-4) Test chemotactic peptide and its derivatives on chemotaxis of MDA-MB-231 cells and protease secretion

Task 2c: (Months 9-15) Refine peptide sequences based on results from task 2b and repeat analysis.

Task 2d: (Month 17) In vitro mutagenesis of OPN chemotactic domain.

Task 2e: (Months 17-24) Express Mutant in osteopontin negative C3H10T1/2 and purify protein

Task 2f: (Months 24-29) Testing the recombinant mutant protein from C3H10T1/2 for biological properties.

Task 2g: (Month 15-24) Test chemotactic response of new CD44 cell lines to chemotactic peptide and its derivatives and test for protease secretion by the CD44 cell lines in response to the peptides

Technical Objective 3 : **Testing the anti-metastatic potential of anti chemotactic domain antibody *in vivo* and testing of novel CD44 cell lines for their metastatic potential *in vivo*.**

Task 3a: (Month 2) Inject mice with cells.
Task 3b: (Month 3) Radiological examination of mice. Finish sacrificing the mice
Task 3c. (Month 4) Histological examination of carcasses.
Task 3d: (Months 12) Inject novel CD44 cell lines in nude mice
Task 3e (Month 13) Radiological examination of mice. Finish sacrificing the mice
Task 3f: (Month 14 -20) histological examination of carcasses

Task 4. (Month 32-36) Finish analysis, write reports and manuscripts

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HYPOTHESIS

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Stress response genes: the genes that make cancer metastasize

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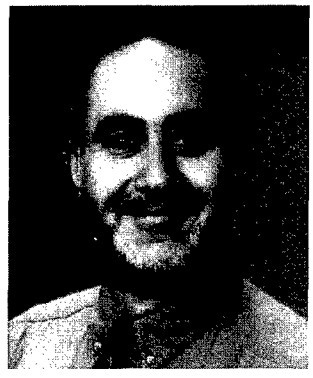

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Abstract Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The topology of cancer spread is mediated by a set of developmentally nonessential genes which are physiologically involved in stress responses, inflammation, wound healing, and neovascularization. The function of these gene products is extensively modified posttranscriptionally. In cancer, metastasis genes are dysregulated at the levels of expression or splicing. These genes constitute a unique group of cancer-related biomolecules.

Key words Cancer · Metastasis · Stress response · Posttranslational modification · Knockout mouse

Introduction

What are the traits that make a killer? This question has intrigued not only fans of detective stories but is also most prominent in the minds of cancer researchers. Here the characteristics of the killer are dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The division of normal cells is tightly controlled by dependence on checkpoints, which are pauses during the cell cycle in which the fidelity of DNA replication and chromosome segregation are monitored. It is regulated by proto-oncogenes, incorporating genes for growth factors, their receptors, and associated intracellular signal transduction molecules. Antagonistic to oncogenes are tumor suppressor genes which normally provide the brakes on cell proliferation. In contrast, cancer is independent of these control mechanisms. Even with defective growth control, however, a cell could never form a tumor of substantial size because, unless it were a germline cell, it would be subject to replicative senescence, an aging process that proceeds with the number of cell divisions and in extreme cases may lead to a state of crisis. A unique role in overcoming replicative senescence is played by the enzyme telomerase, which is expressed in virtually all tumor cells but is ab-

sent from most normal cells. It prevents telomere shortening with increased number of cell divisions, which would eventually cause genomic instability. Mutator genes which encode DNA repair enzymes might be more accurately referred to as meta-oncogenes because their defects give rise to mutations in oncogenes and tumor suppressor genes. Finally, most cells, with the exception of blood and immune cells, grow anchored in their microenvironment whereas cancer cells of particular tissue origin metastasize to specific target organs. The ability of cancer to disseminate throughout the body also sets it apart from benign tumors. However, the classical cancer genes conspicuously do not account for metastasis formation, and current paradigms of cancer have not yet incorporated metastasis genes as a unique group of genes that contributes to the malignant phenotype.

The gene products of stress responses mediate metastasis formation: lessons from knockout mice

The topology of metastasis formation is mediated by the potpourri of homing receptors on the tumor cell surface (Fig. 1) and their ligands and is widely believed to have its physiological correlate in morphogenesis during embryonic development. This would imply that the deficiency of individual metastasis genes should cause defective formation of the relevant target organ. Unexpectedly, knockout mice in which individual genes known to participate in tumor spread were disrupted proved to be fertile and developmentally normal (Table 1). This raises the question: What is the physiological process that goes astray in cancer dissemination?

Despite their diversity, metastasis-associated gene products have several features in common. They comprise a set of genes which physiologically mediate stress responses, including inflammation, wound healing, and neovascularization. Consistently the defects observed in the relevant gene targeted mice are impairments in these areas. This insight resolves some of the paradoxes of metastasis research. In contrast to morphogenesis, invasiveness and tissue damage are in keeping with the normal functions of host defenses that are executed by macrophages and lymphocytes in stress situations. Homing to and expansion in the lymphoid system, typically the first target in metastatic spread, corroborate the notion that cancer metastasis is based on mechanisms normally employed by immunocytes [1]. Differentiation of immune cells proceeds in the context of their tissue of residence, and lymphocytes from Peyer's patches are therefore distinct from cutaneous lymphocytes, and Kupffer cells are distinct from alveolar macrophages. Recognition of topology is encoded in the surface molecules of immune cells, and organ preference by cancer may be derived from this principle.

Table 1 Genes that mediate cancer spread are developmentally nonessential. Cancer dissemination is induced by a group of homing receptors, their ligands, and proteinases in conjunction with their associated signal transduction molecules. These gene products do not play a critical role in organ development or fertility but are necessary for stress responses. Knockout mice have been generated for multiple metastasis associated genes and uniformly show these characteristics. Various integrins have also been linked to metastasis formation but most integrin gene knockouts display developmental defects. This may be due to the loss of multiple receptors after deletion of individual integrin genes. Furthermore, some intergin gene products serve dual roles in stress responses and development (*DTH* delayed type hypersensitivity, *MMP* matrix metalloproteinase, *uPAR* receptor for urokinase-type plasminogen activator)

Gene	Types of cancer	Knockout mouse
Receptors		
uPAR	Prostate cancer, breast cancer [24], gastric carcinoma [25], brain tumors [26]	Defect in leukocyte recruitment and adhesion [8]
CD44	Lymphomas [27], sarcomas [28], colon cancer [29], breast cancer [30]	Excessive granuloma formation [9]
L-selectin	Lymphoma [31]	No DTH to cutaneous antigens [10]
LFA-1	lymphoma [32]	Impaired immune response to alloantigens [11]
ICAM-1	Melanoma [33], lymphoma [34], liver carcinoma [35]	Granulocytosis, diminished DTH, impaired neutrophil homing [12, 13]
IAP (CD47)	Ovarian cancer [36]	Impaired granulocyte activation [14]
Ligands		
Osteopontin	Breast cancer [37], osteosarcoma [38]	Defective wound healing, absence of DTH [15, 16]
Thrombospondin-1	Breast cancer [39], pancreas cancer [40]	Susceptibility to pneumonia [17]
sE-selectin	Gastric cancer [41], breast cancer [42], head and neck cancer [43]	reduced stable adhesion of Leukocytes in inflamed microvasculature [18, 19]
P-selectin	Breast cancer [44], colon cancer [45]	Impaired recruitment of immune cells [20]
Proteinases		
Stromelysin-3 (MMP-3)	Breast cancer [46]	Impaired wound healing [21]
Matrilysin (MMP-7)	Colon cancer [47]	Decreased antimicrobial activity, defective reepithelialization in wounded trachea [22, 23]
Macrophage elastase (MMP-12)	Glioma [48]	Impaired macrophage recruitment [21]

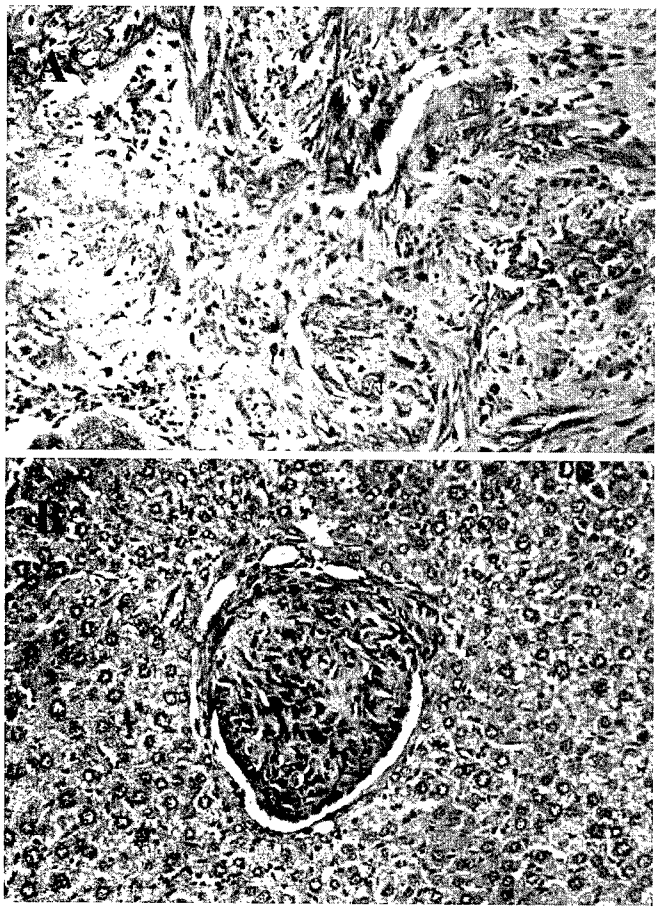

Fig. 1A,B CD44 is essential for metastasis formation by osteosarcoma. C57BL/6 mice with the tm1 point mutation of the *p53* gene are susceptible to osteosarcomas which disseminate to liver and lungs. Shown here are a primary tumor (**A**) and a liver metastasis (**B**). After disruption of both alleles of the CD44 gene metastatic spread is almost completely abrogated while incidence and growth rate of osteosarcomas are unaffected (Weber et al., manuscript in preparation)

The biological activity of metastasis-mediating gene products is extensively regulated by posttranscriptional mechanisms. Collagenases are typically secreted as precursors whose activation requires proteolytic cleavage; collagenase type IV becomes active after cleavage by stromelysin while prostromelysin and interstitial procollagenase are activated by plasmin. Ligands for homing receptors often contain multiple domains. The heparin-binding amino-terminus of thrombospondin stimulates chemotaxis while the carboxy-terminus mediates haptotaxis in an RGD-inhibitable fashion. Comparably, a prerequisite for the interaction of the N-terminal osteopontin domain with integrin receptors is phosphorylation of the cytokine while the C-terminal domain engages variant CD44 by protein-protein interaction. The gene for the homing receptor CD44 contains ten variant exons that can be spliced into the extracellular domain and determine its engagement of various ligands. Differential effects on binding to extracellular matrix and hyaluronate also depend on the glycosylation and sulfation status of CD44. Posttranscriptional modification of function of these molecules may be beneficial in two ways. Activation by mechanisms such as proteolytic cleavage and phosphorylation can be accomplished quickly in stress situations; some of the precursor molecules are widely expressed and can acutely be converted at a site of damage. Also, diversity in structure may encode organ specificity in homing and metastasis formation (a "postal code" of sorts). In clinical diagnosis, tumors that grow in a locally invasive manner but do not form distal metastases, including cases of basalioma, glioblastoma, chondrosarcoma, and myelomonocytic leukemia, are often referred to as semimalignant. The molecular mechanisms of local invasion, however, are distinct from conventional forms of cancer only insofar as their target tissues are identical to the tissues of origin.

In conclusion, the topology of cancer spread is regulated by a set of developmentally nonessential genes that physiologically mediate inflammation, wound healing, and neovascularization. The function of their products is extensively regulated posttranscriptionally. The entity of these genes encodes the repertoire of stress responses which are predominantly executed by macrophages and lymphocytes. Metastasis-associated gene products therefore constitute a unique and essential group of cancer related biomolecules whose functions are distinct from those of growth control or senescence genes.

Regulation and dysregulation of metastasis genes

As in the case of yin and yang, phenomena in biology typically have a counterbalance. This also holds true for the regulation of cell dissemination. While tumor suppressor genes inhibit cell cycle progression and serve as antagonists for oncogenes, the genes that mediate metastatic spread are balanced by metastasis suppressor genes. The derived gene products typically are adhesion molecules that procure cell anchorage and inhibit migration. Expression of L-CAM is inversely correlated with the metastatic potential of various tumor cell lines. Loss of cadherin expression in squamous cell carcinomas of the head and neck, prostate cancer, and cancers of the female reproductive tract is associated with poor differentiation and high invasiveness. E-cadherin can prevent the invasive phenotype in T-lymphoma cells. Proteinases also have their antagonists. Tissue inhibitors of metalloproteinases negatively regulate invasion. Their overexpression reduces metastatic potential whereas antisense RNA enhances the malignant phenotype.

It could be argued that metastasis-associated genes are not, in strict terms, cancer genes because mutations in them have not been linked to the risk of contracting cancer. While it is true that these genes have not yet been observed to be mutated in malignancies as in the case of the classical oncogenes (frequently through point mutations, deletions, frame shifts, or translocations), they are subject to dysregulation. A case in point is the expression of ICAM-1 on melanoma cells, which is an indica-

tor of poor prognosis. Similarly, the homing receptor CD44 is often expressed on cancer cells but not at all on their benign precursors. Alternatively, cancer cells may display splice variants of this receptor which are not detected on their nontransformed counterparts. Therefore aberration of genes for cancer spread occurs frequently at the level of transcription or splicing. Without this dysregulation of gene expression tumors could not become malignant.

Metastasis genes and classical cancer genes: the big picture

Even though uncontrolled growth does not inevitably lead to metastatic spread, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection between mutations of oncogenes or tumor suppressor genes and the expression of genes that mediate tumor dissemination. The molecular basis for this connection is currently largely unknown. Expression of metastasis-specific splice variants of CD44 and the oncogene *ras* are connected in an autocatalytic mode in which *ras* induces promoter activity for CD44 through an AP-1 binding site while transfection of CD44v enhances the expression of *ras*. This mutual induction may contribute to the perpetuation of cell division and spreading which are characteristic of malignancy. Motility-associated cytokines, including type IV collagenases and osteopontin, can also be induced by *ras* and similar relationships may apply for other oncogenes, including v-*mos*, v-*raf*, v-*fes*, and v-*src* [2].

Recent research has identified the genes that underlie the three phenotypic characteristics of cancer and has allowed a distinction between malignant and benign tumors at the molecular level. Only tumors in which the dysregulation of growth is associated with expression of genes whose products mediate dissemination become malignant. This attributes a central role in carcinogenesis to metastasis genes and metastasis suppressor genes. The definition of molecules that are rarely expressed in the healthy adult organism has given rise to the potential emergence of new drug targets. Among them are telomerase, structurally altered oncogene products such as fusion proteins or mutants, and also some of the stress response molecules that mediate metastasis formation. Prominently, blocking the integrin $\alpha_v\beta_3$, which is essential for tumor angiogenesis, has been successful in several experimental systems [3, 4]. Likewise, splice variants of CD44 that mediate dissemination of multiple cancers and are physiologically expressed on immune cells only after antigenic challenge have been targeted in experimental therapy with promising results [5, 6, 7]. Such progress provides the opportunity for a more successful broad attack on the cancer epidemic. As the profile of the killer becomes more refined the prospect for its containment improves.

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Molecular mechanisms of tumor dissemination in primary and metastatic brain cancers

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ABSTRACT: Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. Tumor dissemination distinguishes malignant from benign neoplasms and is mediated by homing receptors, their ligands, and proteinases. The homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases. Its engagement by osteopontin physiologically induces macrophage chemotaxis, a mechanism that may be utilized by metastatic brain tumors in the process of dissemination. In host defense, osteopontin and its receptors, CD44 and integrin $\alpha_V\beta_3$, play key roles in mediating delayed type hypersensitivity responses by activating macrophages to induce Th1 cytokines while inhibiting Th2 cytokines. Other metastasis associated gene products similarly contribute to host defenses. Hence, cancer spread is regulated by a set of developmentally nonessential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization. Function of the relevant gene products is extensively modified post-transcriptionally and their dysregulation in cancer occurs on the levels of expression and splicing. Consistent patterns of organ preference by malignancies of particular tissue origin suggest a necessary connection between loss of growth control and senescence genes and expression of genes mediating the dissemination of tumor cells. © 2000 Elsevier Science Inc.

KEY WORDS: Invasion, Homing receptors, Cytokines, Proteinases, Stress response.

MOLECULAR CHARACTERISTICS OF CANCER

The most prominent feature of malignancy is dysregulated cell cycle progression. Division of cancer cells leads to formation of more cancer cells indicating that the characteristics of transformation originate in genetic changes. The underlying defects causing uncontrolled proliferation are gain of function mutations in oncogenes or loss of function mutations in tumor suppressor genes. However, most somatic cells, with few exceptions such as stem cells, die after a finite number of cell divisions, a phenomenon described as senescence. Replicative senescence begins after fertilization and is genetically dominantly controlled. For cancer to occur, there must be a loss of function in senescence genes or a gain of function in telomerase to give rise to a largely unlimited number of cell divisions. Finally, cancer is distinguished from benign tumors by its faculty to generate metastases. In contrast to earlier models, metastasis formation is a process of active cell migration and invasion rather than the passive dyslocation of cells in the blood or lymph flow. Whether a neoplasm metastasizes and to which target organs is determined by motility associated molecules expressed by the tumor cells.

INVASIVENESS OF BRAIN TUMORS

The brain is unique as a target organ for metastatic growth because it is surrounded by the blood—brain barrier and it lacks lymphatic drainage. Nevertheless, certain malignancies display a preference for dissemination to the central nervous system (CNS). Brain metastases from colon and breast cancers are often single, whereas melanoma and lung cancer have a greater tendency to produce multiple colonies. At autopsy, up to 80% of melanoma patients have CNS lesions [20]. Invasion of brain cancer cells typically proceeds along anatomic structures that are rich in extracellular matrix proteins, including basement membranes of blood vessels and the glial limitans externa [4] and has been attributed to specific motility-associated receptors, their ligands and proteinases [6] (Table 1). Specifically, the homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases [10,12,15]. Its ligand osteopontin has also been described to be secreted by malignant gliomas [5,16,22].

THE PHYSIOLOGIC ROLES OF METASTASIS GENES

To understand the process of metastasis formation we have studied the physiologic importance of the relevant gene products. We have investigated the cytokine osteopontin and its receptor CD44 [25–27]. The engagement of CD44 by osteopontin induces macrophage chemotaxis, a process that may be utilized by metastatic brain tumors in the process of dissemination [26]. Gene-targeted mice deficient in osteopontin or CD44 are fertile and developmentally normal, a trait that is shared by other knockouts for genes that are believed to be important in metastatic spread. Several observations implied that osteopontin may act as a stress

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TABLE 1

METASTASIS-MEDIATING MOLECULES IN BRAIN TUMORS

Tumor	Cytokines	Receptors	Proteinases
Primary brain tumors			
Glioblastoma	Urokinase plasminogen activator, interleukin-8, osteopontin	CD44	Gelatinase-B, active Gelatinase-A, Cathepsin L
Astrocytoma	Hepatocyte growth factor/scatter factor, interleukin-8	c-Met	MT1-MMP, MT2-MMP
Medulloblastoma		polysialylated NCAM	
Metastatic brain tumors			
Melanoma		Neurotrophin receptor	Heparanase
Lung cancer	Urokinase plasminogen activator		
Breast cancer	Urokinase plasminogen activator	Interleukin-6 receptor, CD44	
Prostate cancer		Insulin-like growth factor receptor	
Renal cancer		Interleukin-6 receptor	

Specific receptors, ligands (migration inducing cytokines), and proteinases have been associated with the invasive behavior of individual primary and metastatic brain tumors. MMP, matrix metalloproteinase.

response gene: (1) the osteopontin promoter contains an acute phase responsive element [9] and a phorbol ester responsive element to which the redox sensitive transcription factors Jun and Fos may bind [13], (2) osteopontin expression by T-lymphocytes, macrophages, and osteoclasts does not occur at rest but is activation dependent and is associated with host resistance [13], (3) osteopontin exerts anti-oxidant effects and prevents cell damage in response to a large number of noxious influences [23], (4) the osteopontin gene knockout results in defective wound healing [11]. In host defense, CD44 and its ligand osteopontin play a key role in mediating delayed type hypersensitivity responses by skewing the pattern of cytokines secreted from macrophages to favor the in-

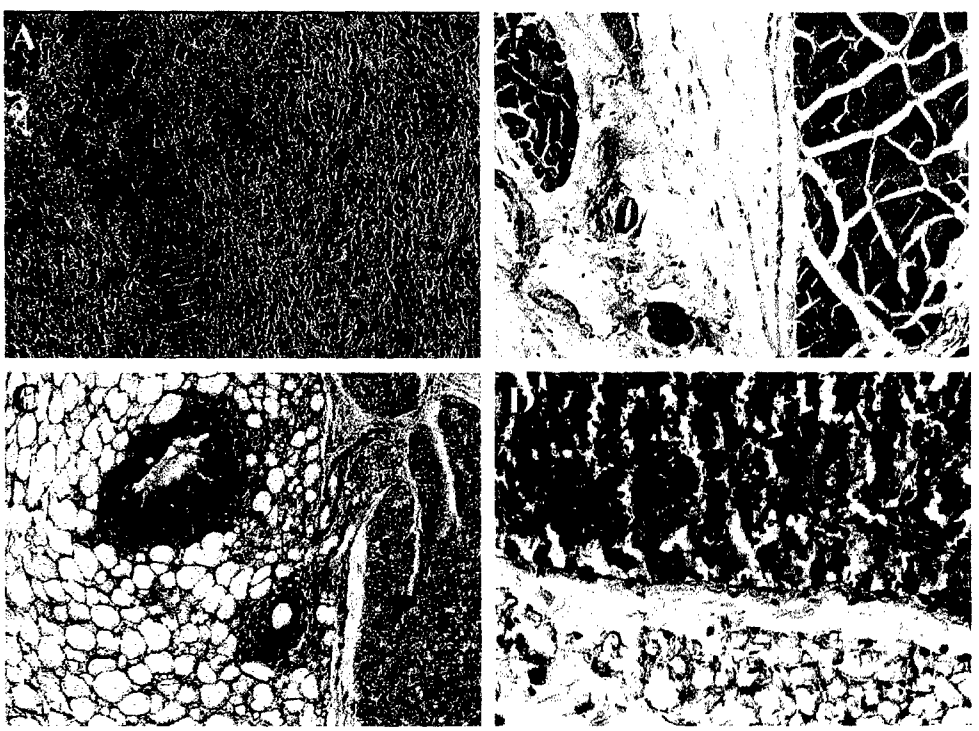

FIG. 1. Absence of a delayed type hypersensitivity response in mice lacking the osteopontin gene. 250 μg polyvinyl pyrrolidone (PVP) in 500 μl phosphate-buffered saline (PBS) was injected subcutaneously into the hind limb of C57BL/6 wildtype mice or gene targeted C57BL/6 $OPN^{-/-}$ mice which do not express the osteopontin gene product. After 5 days, histologic analysis of the injection site was performed; 5-μm serial sections were stained with hematoxilin-eosin stain. (A) Injection of PVP into C57BL/6; (B) injection of PBS (vehicle control) into C57BL/6; (C) injection of PVP into C57BL/6 $OPN^{-/-}$ and (D) injection of PVP in conjunction with 10 μg purified osteopontin into C57BL/6 $OPN^{-/-}$. Original magnification 200×.

TABLE 2
GENES ASSOCIATED WITH MALIGNANCIES

Genes	Function	Examples
Oncogenes	Growth factors	EGF, PDGF
	Growth factor receptors	HER-2, erb-B
	Signal transduction molecules associated with growth factor receptors	Akt, Abl, Ras
Tumor suppressor genes	Receptors	DCC,PTC
	Signal transduction molecules	p53, Rb,APC
Senescence genes	Cell cycle regulators	p53,Rb,p21,Fos
Senescence suppressor genes	Regulators of telomere length	Telomerase
Metastasis genes	Homing receptors and their ligands	CD44, selectins, osteopontin
	Proteinases	MMPs
Metastasis suppressor genes	Adhesion receptors	cadherins, L-CAM, KAI1
	Proteinase inhibitors	TIMPs
Mutator genes	Mismatch repair	MSH, PMS
	Base excission repair	Uracil DNA glycosylase
	Nucleotide excission repair	ERCC
	Repair of double strand breaks	XRCC,RAD50,NSB1

The classical cancer genes (oncogones and tumor suppressor genes) control cell replication. For cancer to occur, additional functions need to be dysregulated: genes that cause cellular senescence have to be inactivated and expression of gene products that mediate metastasis formation is essential. For cell cycle progression and cell dissemination alike, there is a physiologic balance that may be disturbed by excessive activity of promoters or by diminished function of suppressors. Defects in mutator genes give rise to alterations in other cancer-associated genes putting mutator genes into the position of predisposing factors rather than direct contributors to the malignant phenotype.

duction of cellular immunity and to suppress humoral immunity. The interaction of osteopontin with its integrin receptor $a_V\beta_3$ on macrophages stimulates the production of Th1 cytokines while engagement of CD44 by osteopontin concomitantly inhibits the secretion of Th2 cytokines [2]. A classical model of delayed type hypersensitivity is granuloma formation. Foreign body granulomas can be induced by subcutaneous injection of polyvinyl pyrrolidone. After 5 days, control mice display pronounced influx of macrophages and a strong local immune response. In contrast, mice lacking the osteopontin gene due to targeted mutation barely show any immunological reaction to the injection (Fig. 1). In contrast, mice lacking the CD44 gene display excessive granuloma formation following challenge [17] which may reflect combined Th1 and Th2 immunity after engagement of integrin receptors by osteopontin in the absence of ligation of CD44.

Preliminary experiments have suggested that $CD4^+$ T-cells secrete the osteopontin that induces macrophages to selectively promote delayed type immune responses. The observation that macrophages may themselves produce osteopontin after stimulation with lipopolysaccharide raises questions regarding its potential relevance to this process. Macrophage-derived osteopontin is competent for inducing chemotaxis but not delayed type hypersensitivity which may reflect structural differences from the T-cell secreted molecule. In fact, macrophage osteopontin has lost part of its sequence by alternative splicing [Ashkar and Weber, unpublished observations] which could lead to efficient engagement of CD44 with ensuing chemotaxis but to impaired ligation of integrin receptors. Malignant cells often secrete a form of osteopontin that resembles the macrophage-derived protein in that it may be hypophosphorylated or a splice variant that has a deletion in its N-terminal (integrin binding) portion [8] and this molecule may contribute to metastatic spread [25] by inducing tumor cell migration. Concomitantly, tumor-derived modified osteopontin may ligate CD44 on macrophages without engagement of its integrin receptors [19]. This leads to suppression of Th2 cytokines while Th1 cytokines cannot be efficiently secreted since other physiologic inducers of Th1 cytokines are substantially less potent. This form of osteopontin action may represent a mechanism of immune evasion.

Tumor dissemination depends on neovascularization. Physiologically, blood vessel formation may be initiated in two settings. The modeling of the cardiovascular system is largely restricted to early development, while in the healthy adult organism, angiogenesis is a rare occurrence that arises predominantly in healing after tissue damage. Morphogenic and stress induced blood vessel generation are mediated by distinct sets of genes. Several pieces of evidence imply a role for osteopontin and its receptors in the latter form of neovascularization. A splice variant of CD44 is involved in endothelial cell proliferation, migration, and angiogenesis [7, 21]. The integrin $a_V\beta_3$ is of particular importance in angiogenesis due to its selective expression on growing blood vessels. Antagonists of integrin $a_V\beta_3$ promote tumor regression by inhibiting neovascularization [1,3] and angiogenesis induced by bFGF or by TNFα is also inhibitable by a monoclonal antibody to the integrin $a_V\beta_3$. Coordinate expression of β_3-integrins and osteopontin by regenerating endothelial cells [11] and during in vitro blood vessel formation [14] stimulates migration through cooperative mechanisms involving activation of integrin $a_V\beta_3$ ligation by thrombin cleavage of osteopontin [18].

CONCLUSIONS

We conclude, based on our own observations in conjunction with data from the literature, that the topology of cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization and are normally expressed by activated lymphocytes and macrophages [24]. Function of the relevant gene products is extensively modified post-transcriptionally which allows for quick activation in stress situations and may encode organ specificity. This code for targets in the homing process may cause dissemination to distant organs, such as brain metastases in melanoma or lung cancer, or it may lead to locally invasive growth as is the case in malignant glioma or in chondrosarcoma. In both scenarios, locally destructive growth by malignant glioma and brain metastases from distant primary tumors, the mechanism of invasion is determined by engagement of molecules that are physiologically used by macrophages and lymphocytes to enter the central nervous system in the context of host defenses, including infection, inflammation, or ischemia.

Indicative of basic mechanisms of homeostasis in human biology, all groups of genes involved in malignancies consist of promoting and suppressing components. Loss of function in one group or gain of function in the counterbalancing group may each affect the balance of forces and constitute a predisposing factor for malignant growth. Thus, mutations that enhance the function of oncogenes and mutations that inhibit the function of tumor suppressor genes equally pose a risk for uncontrolled growth of the affected cells and similar relationships hold for senescence genes and metastasis genes and their respective suppressors (Table 2). Furthermore, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection among dysregulated cell cycle control genes (gain of function of oncogenes or loss of function of tumor suppressor genes), suppression of senescence genes, and expression of genes mediating the dissemination of tumor cells. Today, the molecular basis for this connection is largely unknown.

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